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# *Xenopus* Drf1, a Regulator of Cdc7, Displays Checkpoint-dependent Accumulation on Chromatin during an S-phase Arrest*

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**We have cloned a *Xenopus* Dbf4-related factor named Drf1 and characterized this protein by using *Xenopus* egg extracts. Drf1 forms an active complex with the kinase Cdc7. However, most of the Cdc7 in egg extracts is not associated with Drf1, which raises the possibility that some or all of the remaining Cdc7 is bound to another Dbf4-related protein. Immunodepletion of Drf1 does not prevent DNA replication in egg extracts. Consistent with this observation, Cdc45 can still associate with chromatin in Drf1-depleted extracts, albeit at significantly reduced levels. Nonetheless, Drf1 displays highly regulated binding to replicating chromatin. Treatment of egg extracts with aphidicolin results in a substantial accumulation of Drf1 on chromatin. This accumulation is blocked by addition of caffeine and by immunodepletion of either ATR or Claspin. These observations suggest that the increased binding of Drf1 to aphidicolin-treated chromatin is an active process that is mediated by a caffeine-sensitive checkpoint pathway containing ATR and Claspin. Abrogation of this pathway also leads to a large increase in the binding of Cdc45 to chromatin. This increase is substantially reduced in the absence of Drf1, which suggests that regulation of Drf1 might be involved in the suppression of Cdc45 loading during replication arrest. We also provide evidence that elimination of this checkpoint causes resumed initiation of DNA replication in both *Xenopus* tissue culture cells and egg extracts. Taken together, these observations argue that Drf1 is regulated by an intra-S-phase checkpoint mechanism that down-regulates the loading of Cdc45 onto chromatin containing DNA replication blocks.**

In eukaryotes, DNA replication is initiated by a multistep process. Early in the $G_1$-phase, replication initiation factors are sequentially assembled onto replication origins to form pre-replicative complexes (pre-RCs).[1] At the core of the pre-RC is the origin recognition complex, a six-subunit protein assembly which is required for the subsequent loading of other pre-RC components, including Cdc6/Cdc18, Cdt1, and the Mcms (1). The second phase of the initiation process involves the transition of the pre-RC to a pre-initiation complex (2). A key determinant for this step is the loading of Cdc45 onto pre-RCs (3, 4). This binding requires the concerted actions of the Mcm10 and Mus101 proteins, and the activities of two types of kinases: the S-phase cyclin-dependent kinases (primarily cyclin E-Cdk2) and Dbf4-Cdc7 (2, 3, 5–10). It has been proposed that the S-phase cyclin-dependent kinases act as global activators of S-phase onset, whereas Dbf4-Cdc7 functions at the level of the individual origins and is therefore required throughout S-phase (11, 12).

The Dbf4-Cdc7 kinase consists of a regulatory subunit, Dbf4, and a catalytic subunit, Cdc7. In yeast, the Cdc7 subunit is present at constant levels throughout the cell cycle, whereas the Dbf4 subunit accumulates only during the $G_1$/S-phase and is then degraded by ubiquitin-mediated proteolysis (13–16). The activation of Cdc7 by Dbf4 leads to the phosphorylation of key substrates within the pre-RC. The best characterized substrate both *in vitro* and *in vivo* is the Mcm2 subunit of the Mcm complex (16–19). It has been suggested that phosphorylation of Mcm2 alters the structure of the pre-initiation complex to induce DNA unwinding (20). Mcm subcomplexes possess DNA helicase activity *in vitro* and may be involved in DNA unwinding during origin firing (21, 22).

In S-phase cells, the presence of stalled replication forks or damaged DNA invokes a checkpoint response that delays entry into mitosis until the defect has been repaired (23–25). These regulatory mechanisms are known as S-M checkpoints. The signal transduction cascades that underlie S-M checkpoints have been well characterized in yeast model systems. A defect is recognized by various sensor proteins, which elicit activation of the effector kinases Chk1 or Cds1 (Rad53 in budding yeast), depending on the nature of the checkpoint-inducing DNA signal. These kinases then phosphorylate key targets within the cell cycle machinery to delay mitosis (24). In vertebrates, homologues of the Rad3 protein include ATR and ATM, both members of the family of phosphatidylinositol 3-kinase-like kinases (26). Evidence suggests that ATR is involved in the detection of replication blocks, whereas ATM is activated in response to DNA damage, leading to activation of Chk1 and Chk2, respectively (27–32). Another protein that has been implicated in the signaling in response to replication blocks is Claspin, which was discovered in *Xenopus* as a Chk1-binding protein that is essential for activation of Chk1 following induction of the replication checkpoint (33).

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[1] The abbreviations used are: pre-RC, pre-replicative complex; XTC, *Xenopus* tadpole cell; BrdUrd, bromodeoxyuridine; RACE, rapid amplification of cDNA ends; GST, glutathione *S*-transferase; Pipes, 1,4-piperazinediethanesulfonic acid; CIB, chromatin isolation buffer; HBS, HEPES-buffered saline.

Recent studies in vertebrate systems have also identified a checkpoint pathway that prevents the onset of DNA replication in the presence of damaged DNA. In *Xenopus*, one of the targets of this checkpoint pathway is the Cdc45 protein. Reconstitution of the DNA damage checkpoint in a *Xenopus* cell-free system identified a pathway that activates ATM in response to DNA containing double-strand breaks (7). ATM, in turn, down-regulates cyclin E-Cdk2 activity, thus preventing the loading of Cdc45 onto chromatin and inhibiting DNA replication. Similarly, Cdc45 is a target of a checkpoint induced by treatment with etoposide, a topoisomerase II inhibitor (34). Activation of this checkpoint, which is mediated by ATR, leads to the down-regulation of Cdc7-associated kinase activity and inhibition of the binding of Cdc45 to chromatin.

In yeast, similar pathways are involved in signaling a third checkpoint, the intra-S-phase checkpoint. This checkpoint inhibits the firing of late replication origins in the presence of stalled replication forks or DNA damage that occurs during S-phase. In budding yeast, both subunits of the Dbf4-Cdc7 kinase undergo Rad53-dependent phosphorylation in response to treatment with hydroxyurea. This phosphorylation releases Dbf4-Cdc7 from chromatin, inhibits its kinase activity, and thereby prevents the loading of Cdc45 (4, 13, 14, 35). In mammalian cells, a recent study showed that mice lacking Cdc7 are embryonic lethal, demonstrating that this kinase is essential for embryonic development (36). Furthermore, when Cdc7 was conditionally removed from an embryonic stem cell line, cells arrested within S-phase with partially replicated DNA. These results support a critical role for the Dbf4-Cdc7 kinase in ensuring the integrity of the DNA throughout the process of replication.

In this study we have cloned a *Xenopus* member of the Dbf4 family, which we have named *Xenopus* Dbf4-related factor 1 (Drf1). The Drf1 protein is not essential for DNA replication or the recruitment of Cdc45 to chromatin during normal S-phase. However, Drf1 displays highly regulated binding to replicating chromatin. Unlike the scenario in yeast and the etoposide-induced pathway in *Xenopus*, we have observed that, following replication fork arrest, Drf1 and Cdc7 accumulate on chromatin. This binding is dependent upon ATR and Claspin, and is abrogated by treatment with caffeine. The loss of Drf1 from chromatin in the presence of caffeine correlates with an increase in Cdc45 loading, which is also observed in aphidicolin-treated extracts lacking ATR or Claspin. Caffeine is also capable of overriding an intra-S checkpoint that prevents further initiation events in the presence of aphidicolin, both in XTC cells and in egg extracts. We have established a biochemical assay for this checkpoint by using alkaline agarose gels and have observed a significant increase in the synthesis of small DNA fragments when extracts are treated with aphidicolin and caffeine. We hypothesize that activation of ATR by stalled replication forks leads to the accumulation of Drf1 on chromatin in a checkpoint-dependent manner. This checkpoint-dependent association of Drf1 with chromatin may play a role in preventing the binding of Cdc45 to chromatin during a replication arrest.

## EXPERIMENTAL PROCEDURES

*Cloning of a Xenopus Drf1 Homologue*—The following oligonucleotides were designed corresponding to sequences in a *Xenopus* expressed sequence tag (accession no. BG408573) from the EMBL sequence library: sense, 5′- gcagcaggacgatgaacccccattggcc-3′; antisense, 5′-ccttc-cgttccgcagcctggatttgggac-3′. A polymerase chain reaction (PCR) using a *Xenopus* cDNA library generated with the Marathon RACE kit (Clontech) served as the template. The 270-bp PCR product was subsequently biotinylated and used as a probe to screen a *Xenopus* oocyte cDNA library in the pAX-NMT vector (37) using the ClonCapture cDNA selection kit (Clontech). The same PCR fragment was radiolabeled and used to isolate the full-length cDNA from the pool of clones enriched for Drf1. Positive clones were verified by PCR, and sequencing of both strands was performed with an ABI model 373 automated sequencer. The GenBank™ accession no. for Drf1 is AY328889.

*Antibodies*—*Xenopus* Cdc7 was amplified by PCR with the following oligonucleotides: 5′-gggaattccatatgagttcgggcgataattcagg-3′ and 5′-act-ggggaattcctaccgcatgtttttaaacagagc-3′ (38). The RACE *Xenopus* cDNA library described above served as the template. The full-length Cdc7 coding sequence was cloned into the *Nde*I and *Eco*RI sites of the pET3-His6X vector, and the resulting plasmid was transformed into Codon Plus *Escherichia coli* cells for expression and purification as described (38). Antibodies were affinity-purified using standard methods with the antigen described above conjugated to CNBr-activated Sepharose 4B (Amersham Biosciences).

Antibodies were raised against an N-terminal fragment of Drf1. The fragment was amplified by PCR using the following oligonucleotides: sense, 5′-gggaattccatatgcagcaggacgatgaacc-3′; antisense, 5′-gccaggt-gaattcctatgtggggctcac-3′. The 1270-bp fragment was cloned into the *Nde*I and *Eco*RI sites of the pET3-His6X vector and expressed in Codon Plus cells. The fusion protein was purified from inclusion bodies as described above for Cdc7. Antibodies were affinity-purified as described above. Affinity-purified antibodies against *Xenopus* Claspin, Chk1, and ATR, and antisera against Orc2 were described previously (33, 39, 40). Antisera against *Xenopus* Cdc45 were generously provided by J. Lee. Monoclonal antibodies against human Mcm2 were obtained commercially (BM28, BD Biosciences). Antibodies recognizing *Xenopus* Mcm4 were kindly provided by J. Blow. Control rabbit IgG was obtained from Zymed Laboratories Inc..

*Xenopus* GST-Mcm2 was cloned into pGEX4T2 vector (Amersham Biosciences), expressed in Codon Plus cells, and purified with GST-agarose. Recombinant geminin was purified as described (33, 39, 40).

*Egg Extracts*—*Xenopus* egg extracts were prepared as described (41). Egg extracts arrested in interphase because of the presence of unreplicated DNA routinely contained 3000 demembranated *Xenopus* sperm nuclei/$\mu$l of extract and 100 $\mu$g/ml aphidicolin. Caffeine was added to a final concentration of 5 mM from a 100 mM solution freshly dissolved in 10 mM Pipes-KOH (pH 7.5).

*Immunodepletion*—Immunodepletions of Chk1 and Claspin from egg extracts were carried out with Affiprep-protein A beads (Bio-Rad) as described previously (33). For immunodepletion of Cdc7 and Drf1, 100 $\mu$l of serum coupled to Affiprep-protein A beads was used per 100 $\mu$l of CSF-arrested extract, during two rounds of depletion. Pre-immune serum served as a control. For immunodepletion of ATR, 25 $\mu$g of antibody coated on protein A-magnetic beads (Dynal) was incubated with extracts on ice for 1 h. The beads were removed with a magnet, and the procedure was repeated.

*Chromatin Isolation*—Egg extracts (100 $\mu$l) containing 3000 sperm nuclei/$\mu$l were overlaid on a 1-ml sucrose cushion containing chromatin isolation buffer (CIB; 20 mM HEPES-KOH (pH 7.6), 1 M sucrose, 80 mM KCl, 25 mM potassium gluconate, and 10 mM magnesium gluconate), and centrifuged at 6100 $\times$ *g* for 5 min. The pellets were washed twice with CIB + 0.5% Nonidet P-40, and centrifuged as above. The chromatin pellets were boiled in 2$\times$ SDS sample buffer and subjected to SDS-PAGE. To elute chromatin-associated proteins, chromatin pellets were prepared from 400 $\mu$l of extract by overlaying 200 $\mu$l on a 1-ml sucrose cushion in duplicate. Samples were centrifuged at 6100 $\times$ *g* for 5 min. Chromatin pellets were washed twice with CIB and then once with CIB + 0.5% Nonidet P-40. The supernatants were removed, and the chromatin pellets were resuspended in 100 $\mu$l of 1 M NaCl, 10 mM HEPES-KOH (pH 7.6). Samples were incubated on ice for 10 min and then centrifuged at 11,700 $\times$ *g* for 5 min. The supernatant contained the proteins eluted from chromatin.

*Replication Assays*—To monitor DNA replication, egg extracts were incubated with sperm nuclei (1000–3000 sperm nuclei/$\mu$l), 0.4 mM $CaCl_2$, 100 $\mu$g/ml cycloheximide, and 1 $\mu$l of [$\alpha$-$^{32}$P]dATP for 90 min at room temperature. The reaction was terminated upon addition of an equal volume of 2$\times$ replication stop buffer (80 mM Tris-HCl (pH 8), 8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, and 0.2% bromphenol blue), and 1 mg/ml proteinase K, followed by incubation at 55 °C for 1 h. Samples were run on 1% agarose gels, dried, and detected by a PhosphorImager (Amersham Biosciences).

*Immunoprecipitations and Kinase Assays*—For immunoprecipitation of Drf1 and Cdc7 from egg extracts, 100 $\mu$l of extract was diluted 3-fold with 10 mM HEPES-KOH (pH 7.6), 150 mM NaCl, 2.5 mM EGTA, 20 mM $\beta$-glycerophosphate, and 0.5% Nonidet P-40, and centrifuged at 11,700 $\times$ *g* for 10 min to pellet nuclei. Supernatants were removed and incubated with 5 $\mu$g of affinity-purified antibody bound to Affiprep-protein A beads for 1 h at 4 °C with rotation. The beads were washed

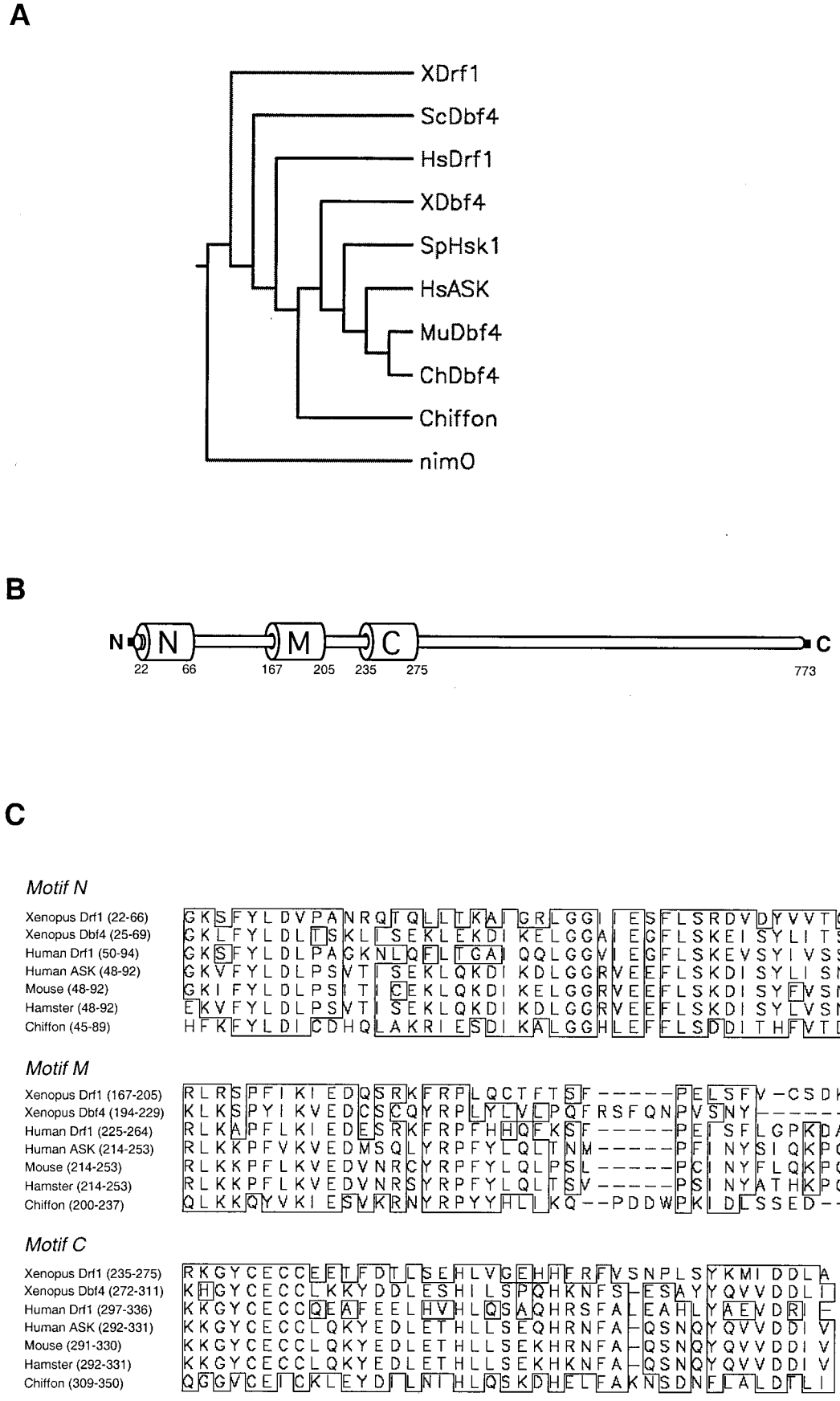


FIG. 1. **Sequence analysis of *Xenopus* Drf1.** *A*, phylogenetic tree displaying the evolutionary relationships among the following Dbf4-like family members: *Xenopus* (XDrf1 and XDbf4), *Saccharomyces cerevisiae* (ScDbf4), human (HsDrf1 and HsASK), *Schizosaccharomyces pombe* (SpHsk1), mouse (MuDbf4), Chinese hamster (ChDbf4), *Drosophila* (Chiffon), and *Aspergillus* (nimO). *B*, schematic of the *Xenopus* Drf1 protein illustrating the three motifs conserved in Dbf4-related proteins. *Numbers* correspond to amino acid position in the sequence of Drf1. *C*, alignment of the motifs in *B* with those in other species. *Numbers* in *parentheses* correspond to amino acid positions in each respective protein.

three times with buffer X (10 mM HEPES-KOH (pH 7.6), 80 mM NaCl, 2.5 mM EGTA, 20 mM β-glycerolphosphate, and 0.1% Nonidet P-40), then once with HBS (150 mM NaCl, 10 mM HEPES-KOH (pH 7.6)). For immunoprecipitation of proteins eluted from chromatin, the supernatant was removed and diluted 8-fold with 50 mM NaCl, 10 mM HEPES-KOH (pH 7.6) and incubated with 5 μg of antibody bound to Affiprep-protein A beads for 1 h at 4 °C with rotation. The beads were washed three times with buffer X, and then once with HBS. For *in vitro* kinase assays, the beads were resuspended in HBS. One half was boiled in 2× SDS sample buffer, subjected to SDS-PAGE, and immunoblotted for Drf1 and Cdc7. The other half was incubated with kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 50 μM ATP) containing [γ-$^{32}$P]ATP and 1 μg of GST-Mcm2. Samples were incubated at room temperature for 15 min with rotation, then boiled in 2× SDS sample buffer, subjected to SDS-PAGE, and detected by a PhosphorImager.

*Cell Culture and Immunofluorescence*—XTC-2 cells were grown on poly-D-lysine-coated coverslips in 61% Leibovitz's (L-15) medium supplemented with 10% fetal calf serum and antibiotics. Caffeine-treated cells were incubated with 5 mM caffeine 90 min prior to aphidicolin treatment, where applicable. The cells were then incubated with 100 μM BrdUrd and 5 μg/ml aphidicolin where indicated. At 5.5 h, cells were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and subjected to indirect immunofluorescence. For BrdUrd visualization, cells were re-fixed with 0.1% formaldehyde and incubated for 10 min in 2 M HCl and 0.1% Triton X-100 at room temperature. The acid was washed away, and the immunofluorescence procedure was repeated with anti-BrdUrd (Roche) as the primary antibody and Texas Red conjugated anti-mouse IgG (Jackson Laboratories) at a 1:500 dilution as the secondary antibody. The coverslips were washed (with 1 μg/ml Hoechst 33258 in the last wash) and mounted onto glass slides with Vectamount (Vector Laboratories). The samples were imaged with a SpotRT CCD camera (Diagnostic Instruments) and analyzed with Adobe Photoshop.

*Alkaline Gel Electrophoresis*—Replication reactions (typically 40 μl of egg extract) were resuspended in 300 μl of StopN (20 mM Tris-HCl (pH 8), 200 mM NaCl, 5 mM EDTA, and 0.5% SDS) containing 2 μg/ml RNase, and digested for 10 min at 37 °C. Proteinase K (200 μg/ml) was added, and samples were incubated for another 30 min at 37 °C. DNA was extracted twice with phenol:chloroform:isoamyl alcohol (Sigma) and then precipitated with ethanol. Pellets were resuspended in 10 μl of 10 mM EDTA, and then diluted with an equal volume of 2× alkaline loading buffer (100 mM NaOH, 2 mM EDTA, 2.5% Ficoll, and 0.025% bromcresol green). Samples were loaded onto gels containing 1% agarose in 50 mM NaCl and 1 mM EDTA, and run overnight in alkaline gel running buffer (50 mM NaOH, 1 mM EDTA). Gels were fixed with 7% trichloroacetic acid, dried, and autoradiographed.

## RESULTS

*Identification of a cDNA Encoding Xenopus Drf1*—A *Xenopus* homologue from the Dbf4 family was cloned by first amplifying a 270-bp sequence with oligonucleotides designed to recognize a *Xenopus* expressed sequence tag with homology to Dbf4 proteins in other species. This fragment was then used to screen a *Xenopus* cDNA library to isolate the full-length open reading frame. Recently, a *Xenopus* Dbf4 sequence has been entered in the GenBank™ data base (accession no. AB095983). Our cloned protein shares significant sequence homology with this protein (28% identical) but represents a second, distinct Dbf4-like polypeptide (Fig. 1*A*). In humans, two Dbf4-like proteins have been identified, both of which activate Cdc7 (19, 42). The cDNA that we have identified in *Xenopus* encodes a 772-amino acid protein that is homologous to both human proteins (32 and 26% identical to human Drf1 and ASK, respectively) but shares a higher identity with Drf1 (Fig. 1*A*). In comparison with other vertebrate Dbf4 proteins, Xdrf1 shares 29% identity with the hamster Dbf4 and 30% identity with the mouse Dbf4. Based on the sequence and the experimental evidence presented below, we believe that we have cloned a Dbf4-related factor, and have named this protein Xdrf1 (referred to hereafter as Drf1).

Three distinct amino acid motifs have been described for the Dbf4 gene family: motifs N, M, and C (43). Motif N shares similarity to a BRCT-like domain and may be functionally

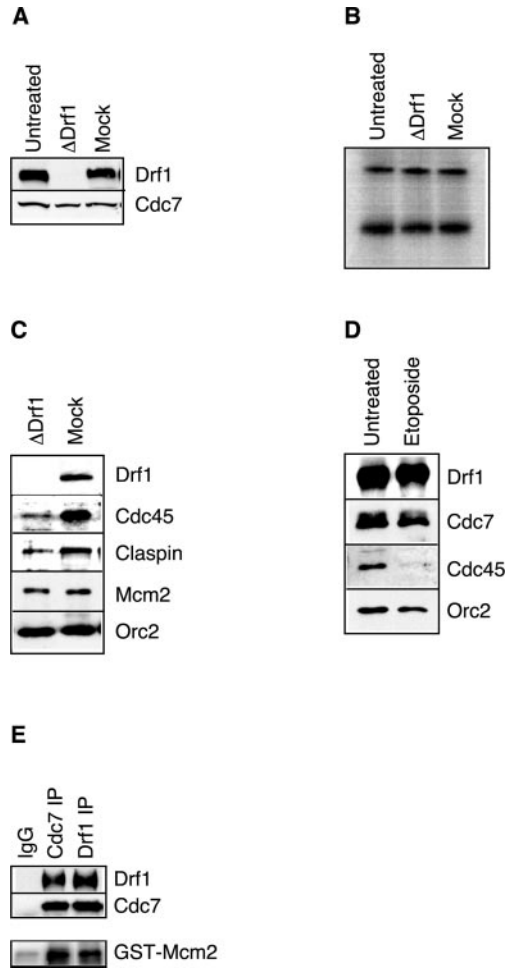


FIG. 2. **Drf1 forms an active kinase with Cdc7 but is not essential for DNA replication.** *A*, Western blot analysis of Drf1 and Cdc7 in untreated, Drf1-depleted, and mock-depleted extracts. *B*, sperm nuclei were incubated in interphase extracts from *A* in the presence of [α-$^{32}$P]dATP for 90 min. DNA replication was determined as described under "Experimental Procedures." *C*, sperm nuclei were incubated for 60 min in mock-depleted and Drf1-depleted extracts. Chromatin fractions were isolated and immunoblotted with antibodies against Drf1, Cdc45, Claspin, Mcm2, and Orc2. *D*, sperm nuclei were incubated in interphase extracts for 100 min in the absence or presence of 30 μM etoposide. Chromatin fractions were isolated and immunoblotted with antibodies against Drf1, Cdc7, Cdc45, and Orc2. *E*, immunoprecipitation of Cdc7 and Drf1 from interphase extracts. Half of the immunoprecipitate was immunoblotted with antibodies against Drf1 and Cdc7. The other half was incubated with GST-Mcm2 and [γ-$^{32}$P]ATP for *in vitro* kinase assays.

important for the replication and DNA damage checkpoints; mutations in this region of the fission yeast homologue, Dfp1/Him1, do not affect the replication functions of the kinase but cause hypersensitivity to drugs that block DNA replication or damage DNA (44). Motif M consists of a proline-rich region, and motif C resembles a CCHH-type zinc finger motif (43). These two regions of Dfp1/Him1 are both necessary and sufficient for full activation of the kinase (44). All three of these motifs are highly conserved in the *Xenopus* Drf1 protein (Fig. 1, *B* and *C*).

*Drf1 Is Dispensable for DNA Replication and Cdc45 Loading*—We raised antibodies against an N-terminal fragment of the *Xenopus* Drf1 protein. This antibody efficiently detects the endogenous Drf1 protein by immunoblotting (Fig. 2*A*). Although the predicted molecular mass of Drf1 is 85 kDa, we found that the protein detected by two different antibodies raised against Drf1 migrated more slowly, at ~150 kDa. Upon removal of Drf1 with anti-Drf1 antibodies, we observed that a

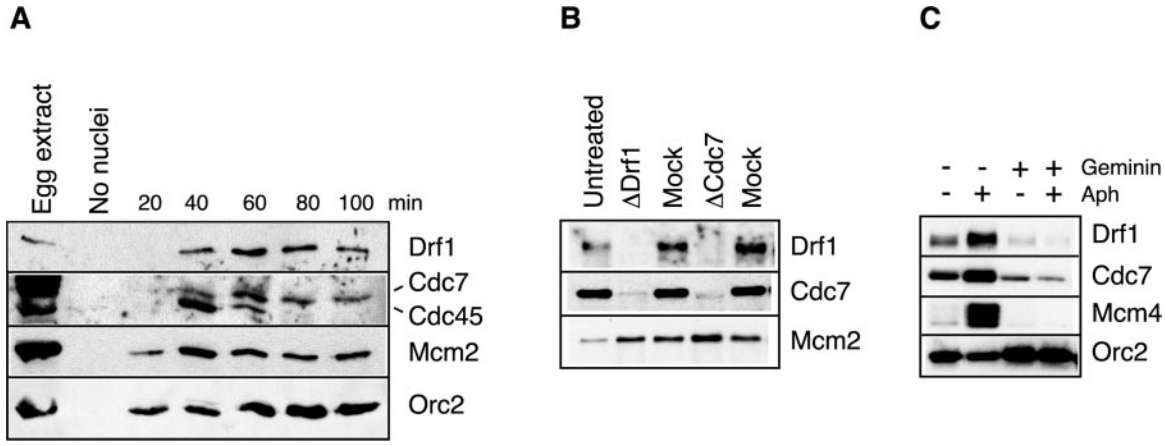


FIG. 3. **Drf1 is a chromatin-binding protein.** *A*, whole egg extract and chromatin fractions isolated at various times (minutes) following addition of sperm chromatin to interphase extracts were immunoblotted with antibodies against Drf1, Cdc7, Cdc45, Mcm2, and Orc2. The *second lane* depicts a mock chromatin fraction from an extract lacking DNA. *B*, mock-depleted, Drf1-depleted, and Cdc7-depleted extracts were incubated with sperm nuclei for 100 min. Subsequently, chromatin fractions were immunoblotted with antibodies against Drf1, Cdc7, and Mcm2. *C*, sperm nuclei were incubated in egg extracts in the absence and presence of aphidicolin (*Aph*) and geminin, as indicated. After 100 min, chromatin fractions were immunoblotted with antibodies against Drf1, Cdc7, Mcm4, and Orc2.

significant amount of Cdc7 remained in the Drf1-depleted extract. Possible explanations are that there is a pool of free Cdc7 or that a second Dbf4-like protein may also form a complex with Cdc7, as is the case in human cells. To determine whether Drf1 is required for DNA synthesis, we depleted Drf1 from egg extracts and monitored the extent of DNA replication. We observed no defect in DNA replication in the absence of Drf1 compared with the mock-depleted or untreated extracts (Fig. 2*B*). This result contrasts with the nearly complete inhibition of replication that has been reported for depletion of the *Xenopus* Cdc7 protein (9, 38). It is possible that our antibodies failed to completely deplete Drf1 from the extracts, thereby allowing some residual kinase to promote DNA replication. However, immunoprecipitation of Drf1 from a Drf1-depleted extract failed to detect any Drf1 protein (data not shown). Hence, our results suggest that either Drf1 is not the regulatory subunit of Cdc7 that is essential for DNA replication or, alternatively, that when Drf1 is depleted from extracts, the replication function of the kinase is performed by a Dbf4-Cdc7 complex.

In yeast, both the Dbf4 and Cdc7 subunits are required for the loading of Cdc45 onto chromatin. In *Xenopus*, a similar requirement has been shown for Cdc7 (45). To determine whether depletion of Drf1 affects the ability of Cdc45 to bind to chromatin, we analyzed the chromatin fraction of Drf1-depleted extracts that had been incubated with sperm nuclei for 60 min (Fig. 2*C*). Although we observed a significant reduction in the levels of chromatin-bound Cdc45 in the Drf1-depleted extracts compared with the control extract, Cdc45 was nonetheless still capable of binding to chromatin. Consistent with this observation, Claspin, which is dependent on Cdc45 for its recruitment to chromatin during interphase (39), was also bound to chromatin in the absence of Drf1, albeit at lower levels. As expected, the binding of components of the pre-RC, including Orc2 and Mcm2, was not dependent on Drf1. These results are consistent with the hypothesis that another member of the Dbf4 family must contribute to the replication function of the Cdc7 kinase.

A recent report identified a *Xenopus* Dbf4 protein as the target of an etoposide-induced checkpoint (34). In the presence of etoposide, it was observed that Dbf4 failed to bind to chromatin, resulting in the loss of Cdc45 loading. We tested whether we could also detect the loss of chromatin binding of Drf1 in the presence of etoposide (Fig. 2*D*). Although etoposide efficiently inhibited DNA replication (data not shown), we failed to observe any change in the chromatin binding of Drf1. However, the chromatin binding of Cdc45 was inhibited by etoposide, consistent with the published results. Collectively, these results support the conclusion that Drf1 constitutes a second member of the Dbf4 family in *Xenopus laevis*.

Given the different properties of *Xenopus* Dbf4 and Drf1, we verified that Drf1 is indeed a regulatory subunit of the Cdc7 kinase. Using Drf1 and Cdc7 antibodies, we tested whether these proteins could be co-immunoprecipitated with each other from *Xenopus* egg extracts (Fig. 2*E*, *upper panel*). We found that Drf1 and Cdc7 formed a stable complex that could be immunoprecipitated with antibodies against either subunit. This complex also formed an active kinase that readily phosphorylated GST-Mcm2 *in vitro* (Fig. 2*E*, *bottom panel*). These results confirm that the Drf1-Cdc7 complex has kinase activity.

*Drf1 Binds to Chromatin in a Regulated Manner*—We next characterized the binding of Drf1 to chromatin. As was reported for Cdc7 (9), Drf1 binds to chromatin after Mcm2 and around the same time as Cdc45 (Fig. 3*A*). Because both Drf1 and Cdc7 bind to chromatin at the onset of S-phase, we tested whether each subunit requires the other to bind to chromatin (Fig. 3*B*). In Drf1-depleted extracts, Cdc7 binding was barely detectable. Likewise, in Cdc7-depleted extracts, we failed to observe any chromatin-bound Drf1. These observations suggest that Drf1 and Cdc7 bind to chromatin as a complex or indirectly stabilize each other on chromatin. Furthermore, when extracts were treated with geminin, an inhibitor of Cdt1-dependent Mcm loading (46), the binding of both Drf1 and Cdc7 to chromatin was strongly inhibited (Fig. 3*C*). This finding indicates that binding of Drf1 and Cdc7 is dependent upon the pre-RC.

*Chromatin Binding of Drf1 and Cdc7 following Aphidicolin Treatment Is Checkpoint-regulated*—Because Cdc7 and Dbf4 are regulated by the DNA replication checkpoint in *Xenopus* and in yeast, we tested whether Drf1 may also have a checkpoint-specific function. In the presence of aphidicolin, which induces a block to replication, it has been reported that the binding of Cdc7 to chromatin is increased (45). We observed a similar increase in the levels of Drf1 following incubation of sperm nuclei in extracts in which the replication checkpoint was activated by aphidicolin (Fig. 4*A*). This increase is also sensitive to geminin, which is consistent with a requirement for initiation to occur to establish the replication checkpoint induced by aphidicolin (Fig. 3*C*). However, the increase in Drf1 and Cdc7 binding to chromatin could simply be a consequence of the increased number of replication forks that stall in response to aphidicolin. In this case, we would predict that the

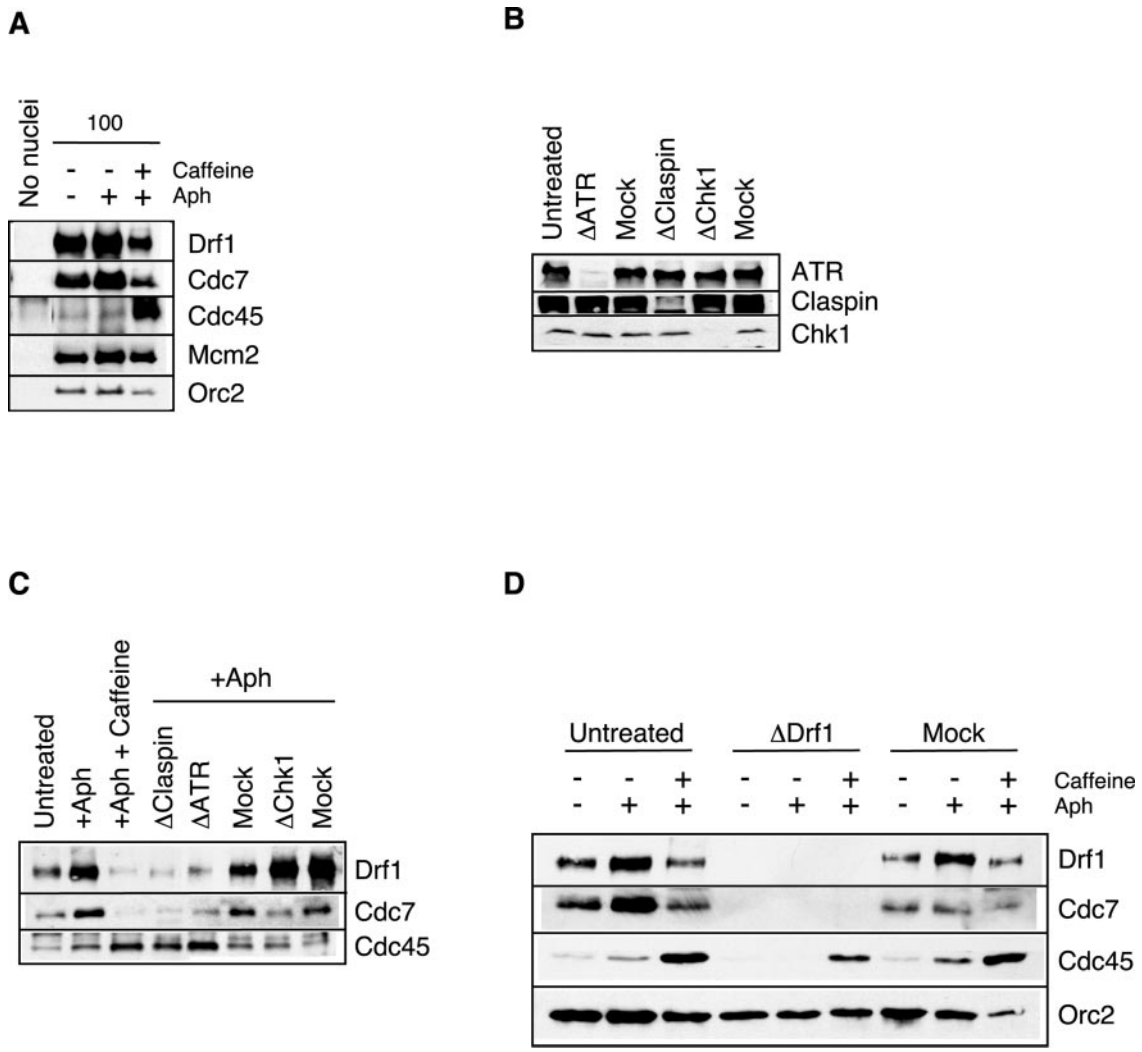


FIG. 4. **Regulation of the binding of Drf1 and Cdc7 to chromatin.** *A*, sperm nuclei were incubated for 100 min in interphase extracts with no drug, aphidicolin (*Aph*), or aphidicolin plus caffeine. Chromatin fractions were immunoblotted for Drf1, Cdc7, Cdc45, Mcm2, and Orc2. *B*, untreated, mock-depleted, ATR-depleted, Claspin-depleted, and Chk1-depleted extracts were immunoblotted with anti-ATR, anti-Claspin, and anti-Chk1 antibodies. *C*, the extracts described in *B* were incubated with sperm nuclei in the presence of no drug, aphidicolin, or aphidicolin plus caffeine for 100 min. Chromatin was isolated and immunoblotted with antibodies against *Xenopus* Drf1, Cdc7, and Cdc45. *D*, interphase, Drf1-depleted, and mock-depleted extracts were incubated with sperm nuclei and either no drug, aphidicolin, or aphidicolin plus caffeine for 100 min. Chromatin fractions were immunoblotted with antibodies against Drf1, Cdc7, Cdc45, and Orc2.

chromatin binding of Drf1 and Cdc7 would be independent of the checkpoint signaling pathways. To test this possibility, we treated extracts with aphidicolin and caffeine, an inhibitor of ATM and ATR, to determine whether inhibition of checkpoint signaling affects the levels of Drf1 and Cdc7 on chromatin. We found that the levels of both Drf1 and Cdc7 on chromatin were significantly reduced in the presence of caffeine (Fig. 4*A*). This behavior is in stark contrast to the regulation of Cdc45. A slight increase in chromatin binding of Cdc45 was observed in the presence of aphidicolin, but the association of Cdc45 with chromatin was dramatically up-regulated upon addition of caffeine (Fig. 4*A*). This observation suggests that Cdc45 loading may be suppressed by a checkpoint pathway that is activated by a replication block.

To characterize further the checkpoint-dependent chromatin binding of Drf1 and Cdc7, we tested whether this binding requires specific components of the checkpoint-signaling pathway. For this purpose, we depleted ATR, Claspin, or Chk1 from egg extracts (Fig. 4*B*). Sperm nuclei and aphidicolin were added to these depleted extracts, and chromatin fractions were immunoblotted for Drf1 and Cdc7. In both the ATR-depleted and Claspin-depleted extracts, we found that Drf1 and Cdc7 levels were reduced as compared with the mock-depleted controls and were very similar to the levels observed in extracts treated with both aphidicolin and caffeine (Fig. 4*C*). In contrast, when Chk1 was depleted, there was no effect on the chromatin binding of either Drf1 or Cdc7. These results suggest that upstream components of the replication checkpoint pathway are required for the recruitment or stability of Drf1 and Cdc7 on chromatin following aphidicolin treatment, yet the chromatin binding of Drf1 and Cdc7 is not dependent on the effector kinase Chk1. Another significant finding involves the effect of ATR and Claspin depletion on Cdc45. When extracts were treated with aphidicolin in the absence of ATR or Claspin, Cdc45 levels on chromatin increased significantly, similar to those observed in the presence of both aphidicolin and caffeine (Fig. 4, *A* and *C*). In contrast, depletion of Chk1 had no effect on Cdc45. From these results we conclude that the regulated chromatin binding of Drf1, Cdc7, and Cdc45 during a replication block is dependent upon ATR and Claspin.

These findings suggest that a replication checkpoint pathway may respond to stalled replication forks by suppressing further loading of Cdc45 at these forks or at other origins. To test whether Drf1 plays a role in preventing additional Cdc45

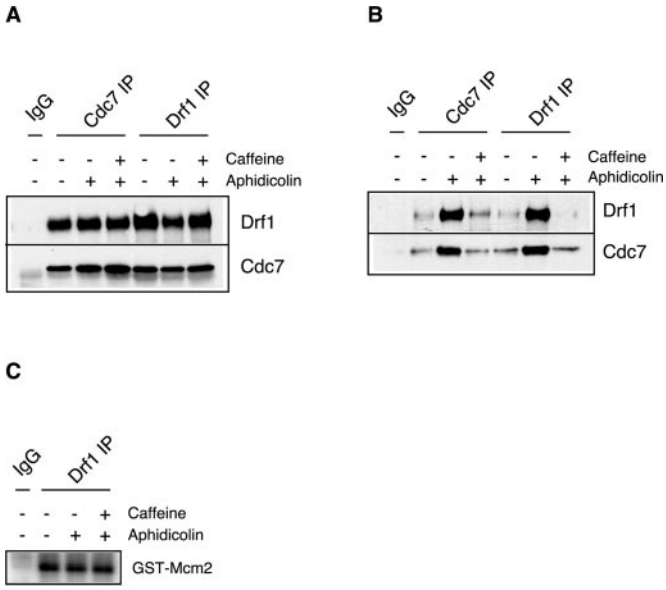


FIG. 5. **The kinase activity of Drf1-Cdc7 is not affected by aphidicolin treatment.** Interphase extracts were incubated without aphidicolin, with aphidicolin, and with aphidicolin plus caffeine. The extracts (*A*) and chromatin fractions isolated from these extracts (*B*) were immunoprecipitated (*IP*) with control, anti-Cdc7, and anti-Drf1 antibodies. The immunoprecipitates were then probed for Cdc7 and Drf1 by immunoblotting. *C*, the kinase activity of immunoprecipitates from *A* was tested *in vitro* using GST-Mcm2 as a substrate.

from binding to chromatin, we depleted Drf1 from egg extracts and then examined the levels of chromatin-bound Cdc45 in these extracts in the presence of no drug, aphidicolin, or aphidicolin and caffeine (Fig. 4*D*). In mock-depleted extract, low levels of Cdc45 were detected in the absence and presence of aphidicolin, whereas a significant increase in Cdc45 loading was observed in the presence of both aphidicolin and caffeine. However, only a small increase in the binding of Cdc45 occurred in Drf1-depleted extracts that were treated with both aphidicolin and caffeine. These findings imply that Drf1 is involved in regulating Cdc45 loading during a replication block.

*Regulation of Drf1-Cdc7 Kinase Activity during the Replication Block*—In *Xenopus* egg extracts, treatment with the DNA damaging agent etoposide disrupts the interaction between Dbf4 and Cdc7. This treatment therefore inactivates the kinase and prevents the binding of Cdc45 to chromatin (34). To determine whether Drf1 and Cdc7 are regulated in a similar fashion in response to aphidicolin, we tested whether both subunits could be co-immunoprecipitated with each other in the extract and on chromatin. In both fractions, Drf1 and Cdc7 co-immunoprecipitated with each other in the presence of aphidicolin (Fig. 5, *A* and *B*). This observation suggests that the Drf1-Cdc7 complex is maintained during a replication block.

We next measured the kinase activity associated with Drf1 that had been immunoprecipitated from either the extract or chromatin fractions, using GST-Mcm2 as a substrate. In the extract, no obvious change in the kinase activity was observed in response to aphidicolin or aphidicolin plus caffeine (Fig. 5*C*). Similar results were observed when the kinase activity of the chromatin-eluted Drf1-Cdc7 complex was measured (data not shown). Taken together, these results indicate that the aphidicolin-induced checkpoint does not lead to a readily discernible inhibition of the kinase activity of Drf1-Cdc7 under these assay conditions.

*A Caffeine-sensitive Checkpoint Inhibits Replication in XTC Cells during Aphidicolin Arrest*—Our chromatin binding data suggest that an aphidicolin-induced, caffeine-sensitive checkpoint regulates the binding of Drf1 and Cdc7 to chromatin. The aphidicolin-dependent increase in the chromatin binding correlates with a suppression of Cdc45 that is reversed upon abrogation of the checkpoint with caffeine. These observations suggest that Drf1 is a target of an intra-S checkpoint that regulates the loading of Cdc45. To investigate whether an intra-S checkpoint exists in *Xenopus* cells, we treated *Xenopus* tadpole cells (XTC cells) with BrdUrd and monitored its incorporation in asynchronous cells after a 5.5-h incubation with aphidicolin or with aphidicolin and caffeine. Treatment with caffeine alone or $Me_2SO$ served as controls. We then counted the percentage of the total number of cells that exhibited punctate BrdUrd staining, an indicator that these cells were in S-phase. As expected, we observed a dramatic decrease in the number of BrdUrd-positive S-phase cells after treatment with aphidicolin (Fig. 6, *A* and *B*). When caffeine was added together with aphidicolin, the percentage of cells increased significantly ($p < 0.01$), approximately to levels observed in untreated cells or cells treated with caffeine alone. Similar results were recorded when we calculated the percentage of punctate BrdUrd cells over the total number of BrdUrd-positive cells (data not shown). However, the intensity of the BrdUrd labeling in the nuclei of cells treated with both caffeine and aphidicolin was strongly reduced compared with untreated S-phase cells (Fig. 6*A*, compare *left* and *right panels*). These observations indicate that caffeine mostly does not reverse the inhibitory effect of aphidicolin on DNA polymerase activity to allow extensive elongation and completion of DNA synthesis. Instead, the enhanced incorporation of BrdUrd in the presence of caffeine may reflect the abrogation of a checkpoint that suppresses origin unwinding and early events associated with the initiation of DNA replication.

*Caffeine-sensitive Inhibition of Nascent Strand DNA Synthesis in Xenopus Extracts*—We next sought a biochemical assay to determine whether an analogous intra-S checkpoint may operate in *Xenopus* egg extracts. It has been reported that in the presence of lower doses of aphidicolin (10 $\mu$g/ml), small DNA fragments ranging in size from 0.1 to 1 kb accumulate during a replication block. These fragments can be detected in alkaline agarose gels (47). Using this dose of aphidicolin, we were able to detect a low level of DNA fragments that accumulated within this same size range (Fig. 7*A*). We hypothesized that, if a checkpoint functions to suppress origin firing in the presence of aphidicolin, perhaps this checkpoint may be reversed by caffeine, thereby allowing more synthesis of these small DNA fragments. Indeed, in extracts treated with both aphidicolin and caffeine, we observed a significant increase in the accumulation of these small fragments compared with treatment with aphidicolin alone (Fig. 7*A*). Although some of the fragments appeared longer than in the aphidicolin-blocked sample, the bulk of DNA synthesized in the presence of caffeine remained within the same size range. This observation suggests that aphidicolin still prevents elongation in the presence of caffeine, but some aspect of initiation is under checkpoint control.

## DISCUSSION

In this work, we have cloned and characterized *Xenopus* Drf1, a member of the Dbf4 family of proteins. Based on published results that describe another Dbf4-related homologue in *Xenopus* (34) and its reported sequence, Drf1 represents a second, distinct protein. Thus, *Xenopus* is the second vertebrate species, after humans, to have two Dbf4 homologues. In fission yeast, two Dbf4-like complexes have also been identified: Dfp1-Hsk1 and Spo6-Spo4. Both complexes form active kinases, but their functions are quite divergent. Dfp1-Hsk1, which is most analogous to mammalian Dbf4-Cdc7, is essential for DNA rep-

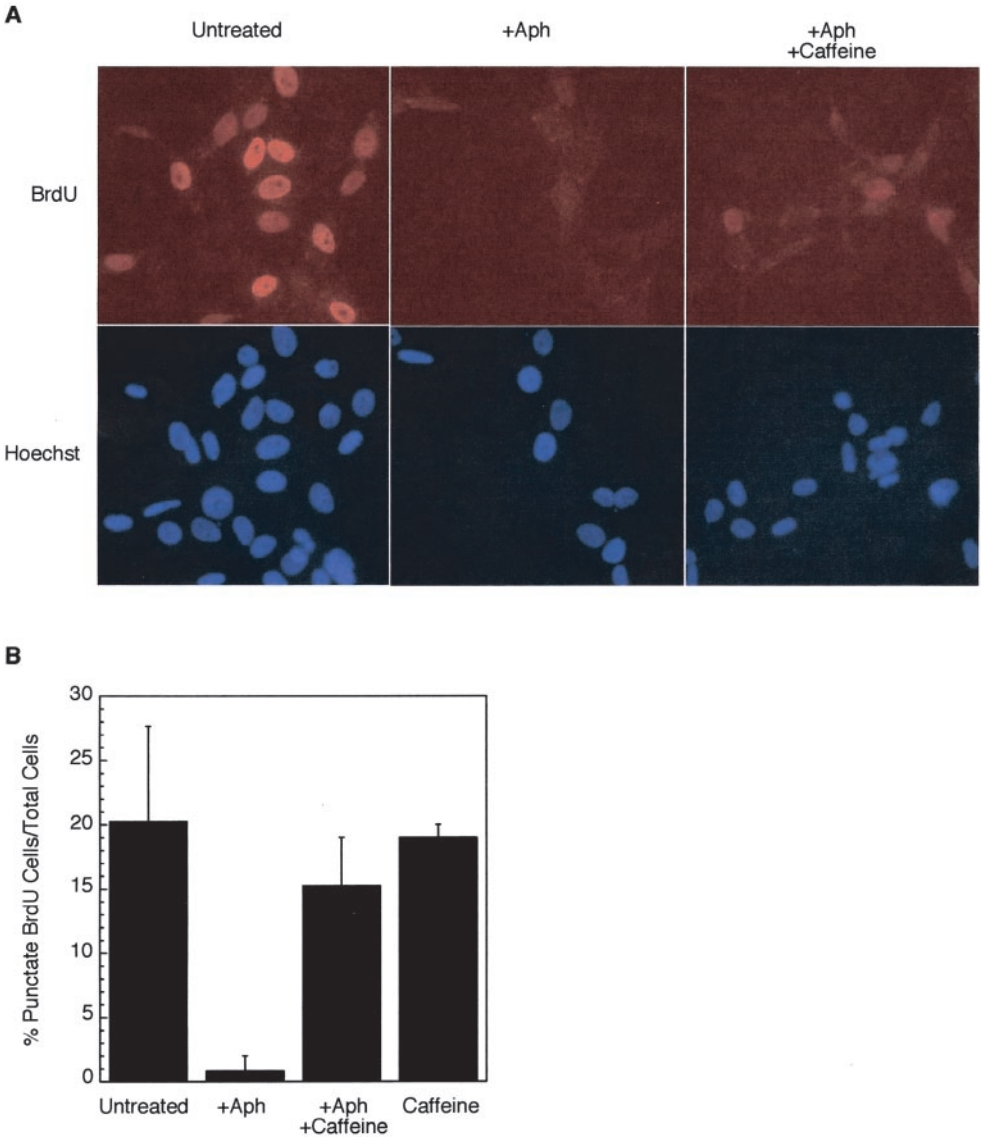


FIG. 6. **Caffeine induces some BrdUrd (*BrdU*) incorporation in aphidicolin (*Aph*)-treated XTC cells.** *A*, immunofluorescence of BrdUrd-labeled XTC cells treated with $Me_2SO$, aphidicolin alone, or aphidicolin and caffeine for 5.5 h. BrdUrd incorporation was visualized using an anti-BrdUrd antibody (*upper panels*), and Hoechst dye was used for nuclear DNA staining (*lower panels*). *B*, quantification of cells in *A* expressed as the percentage of cells with punctate BrdUrd staining over the total number of cells.

lication (15, 48, 49), whereas Spo6-Spo4 is involved in progression through the second meiotic division (50). In humans, the two Dbf4-like proteins (ASK and Drf1) regulate the same catalytic partner, Cdc7. Both complexes are activated during S-phase. Although ASK-Cdc7 is essential for DNA replication (19, 51), it is not known what role human Drf1-Cdc7 plays during this phase of the cell cycle.

We have found that the *Xenopus* Drf1 homologue is dispensable for DNA replication and loading of Cdc45 onto chromatin. It is possible that both Drf1-Cdc7 and Dbf4-Cdc7 complexes contribute to Cdc45 loading, and therefore a defect in replication would be observed only by depleting both regulatory subunits. This possibility would be consistent with our findings that Cdc45 levels on chromatin are reduced in Drf1-depleted extracts, and published results that depletion of the Cdc7 subunit is sufficient to block DNA replication and Cdc45 loading (9, 38). Although Drf1 may not be essential for DNA replication in *Xenopus*, it does bind to chromatin in a highly regulated manner. In particular, it associates with chromatin at S-phase onset at around the same time as Cdc7 and Cdc45. The binding of Drf1 to chromatin is sensitive to geminin, as was shown previously for Cdc7 (45), implying that binding of Drf1 and Cdc7 occurs once the Mcm complex has been recruited to the pre-RC. Although the exact function of chromatin-bound Drf1 and Cdc7 is not known, the two subunits form a complex on chromatin that exhibits kinase activity toward Mcm2 *in vitro*.

Upon further characterization of Drf1, we found that the chromatin binding properties of this protein are regulated during a replication block. Whereas treatment with hydroxyurea causes the budding yeast Dbf4-Cdc7 complex to dissociate from chromatin (13), a replication block induced by aphidicolin results in the accumulation of both *Xenopus* Drf1 and Cdc7 on chromatin. This binding is sensitive to caffeine and diminished in extracts lacking ATR or Claspin. These observations argue against the model that Drf1 and Cdc7 remain on chromatin simply because of the presence of stalled replication forks. Instead, these results suggest that Drf1 and Cdc7 are either actively recruited to or stabilized on chromatin by signaling components of the caffeine-sensitive replication checkpoint. Importantly, we found that depletion of Drf1 substantially reduced the effect of caffeine on Cdc45 loading, suggesting that, although Cdc45 can bind to chromatin in a Drf1-depleted ex-

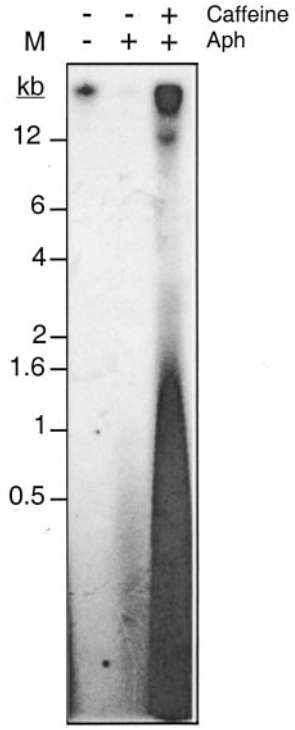


FIG. 7. **Caffeine-sensitive accumulation of nascent DNA during replication arrest.** DNA from untreated extracts (control, *lane 1*) or extracts treated for 100 min with 10 $\mu$g/ml aphidicolin (*Aph*, *lane 2*) or aphidicolin and 5 mM caffeine (*lane 3*) was isolated and run on a 1% agarose gel under alkaline conditions. *M*, $^{32}$P-labeled 1-kb DNA marker. For the *first lane*, one tenth as much sample was electrophoresed as in the *other lanes*.

tract to allow normal replication to proceed, the suppression of Cdc45 by checkpoint activation involves Drf1. One possible model is that the Drf1-Cdc7 kinase on chromatin phosphorylates different targets under checkpoint conditions than during DNA replication, resulting in the inhibition of further Cdc45 loading. Alternatively, Drf1 and Cdc7 may simply be sequestered on chromatin in a manner that contributes to the lack of Cdc45 loading at sites of initiation. This concept is consistent with the model proposed by Edwards *et al.*, in which Cdc7 can interact with Mcms throughout broad regions of chromatin outside of the initiation sites (52).

The activation of a caffeine-sensitive replication checkpoint that targets the chromatin binding of Cdc45 shares certain similarities with the DNA damage pathways previously reported in *Xenopus* cell-free extracts. Treatment with etoposide, an inhibitor of topoisomerase II, results in activation of ATR, which leads to disruption of the Dbf4-Cdc7 complex and inhibition of its kinase activity. This inhibition prevents the loading of Cdc45 and entry into S-phase (34). Interestingly, Dbf4 dissociates from chromatin under these conditions, but Cdc7 remains bound. In contrast, Drf1 remains associated with chromatin in the presence of etoposide, suggesting that its regulation in response to DNA damage differs from that of Dbf4.

In the presence of aphidicolin, limited origin firing and elongation can occur before replication forks stall (10, 47). These arrested replication structures are known to activate a checkpoint pathway that activates ATR, Claspin, and Chk1 to inhibit entry into mitosis (28–31, 33). However, results from yeast and mammalian systems have also identified an intra-S checkpoint pathway that functions to prevent the initiation of replication at late origins in the presence of stalled replication forks (53–55). This pathway is also caffeine-sensitive and involves several components of the S-M checkpoint, suggesting that activation of the replication checkpoint has multiple cell cycle targets. Our data from XTC cells identified a similar caffeine-sensitive intra-S checkpoint that is activated by aphidicolin. Only very low levels of BrdUrd labeling could be detected when cells were treated with aphidicolin, but distinctly punctate BrdUrd labeling was observed when caffeine was also added. It is important to note that the overall intensity of the BrdUrd labeling was not nearly as high as in the untreated nuclei, consistent with continued inhibition of DNA polymerases. Nevertheless, these results suggest that some aspect of DNA unwinding is inhibited by the replication checkpoint pathway in the presence of incompletely replicated DNA.

Using alkaline agarose gels, we have established a biochemical assay for the checkpoint-dependent block to replication in response to aphidicolin in egg extracts. Consistent with our observations in XTC cells, treatment with aphidicolin and caffeine allowed the synthesis of short nascent DNA strands. These results support the hypothesis that an intra-S checkpoint operates in egg extracts to prevent further initiation events in the presence of stalled replication forks. One attractive hypothesis is that, in response to aphidicolin, ATR and Claspin are activated, leading to bifurcating signaling events. One pathway leads to activation of Chk1 and inhibition of Cdc2. The other pathway would lead to the accumulation of Drf1-Cdc7 kinase on chromatin. Abrogation of this pathway by caffeine would then allow Drf1-Cdc7 to participate in the loading of Cdc45 onto chromatin. This process could involve either new origin firing at unreplicated sites or resumption of replication within regions in which origins have already fired. Montagnoli *et al.* (42) have proposed that the human Drf1 might be a specific activator of Cdc7 to fire late origins selectively. Although late origins have not been characterized in the *Xenopus* egg extract system, our results nonetheless are consistent with the possibility that Drf1 plays some role in origin utilization during a DNA replication block.

Another related possibility is that Drf1-Cdc7 acts to ensure proper resumption of DNA replication upon recovery from the arrest. In fission yeast, *dfp1* and *hsk1* mutants exhibit severe defects in recovery from hydroxyurea arrest, consistent with a role in re-starting stalled replication forks (15, 56). In *Xenopus*, ATR and Claspin may participate in the recovery process by recruiting or stabilizing Drf1 and Cdc7 on chromatin. Chromatin-bound Cdc7 has been shown to be capable of supporting DNA replication (9). Thus, when the block to replication is removed, the kinase is poised to load significant amounts of Cdc45 either at origins or at stalled forks to quickly re-initiate and complete DNA synthesis. In both egg extracts and XTC cells, abrogation of this checkpoint could lead to premature re-starting of replication forks and DNA unwinding even in the presence of aphidicolin.

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